

The Maximum Activities of Hexokinase, Phosphorylase, Phosphofructokinase, Glycerol Phosphate Dehydrogenases, Lactate Dehydrogenase, Octopine Dehydrogenase, Phosphoenolpyruvate Carboxykinase, Nucleoside Diphosphatekinase, Glutamate–Oxaloacetate Transaminase and Arginine Kinase in Relation to Carbohydrate Utilization in Muscles from Marine Invertebrates

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1. Comparison of the activities of hexokinase, phosphorylase and phosphofructokinase in muscles from marine invertebrates indicates that they can be divided into three groups. First, the activities of the three enzymes are low in coelenterate muscles, catch muscles of molluscs and muscles of echinoderms; this indicates a low rate of carbohydrate (and energy) utilization by these muscles. Secondly, high activities of phosphorylase and phosphofructokinase relative to those of hexokinase are found in, for example, lobster abdominal and scallop snap muscles; this indicates that these muscles depend largely on anaerobic degradation of glycogen for energy production. Thirdly, high activities of hexokinase are found in the radular muscles of prosobranch molluscs and the fin muscles of squids; this indicates a high capacity for glucose utilization, which is consistent with the high activities of enzymes of the tricarboxylic acid cycle in these muscles [Alp, Newsholme & Zammit (1976) *Biochem. J.* **154**, 689–700]. 2. The activities of lactate dehydrogenase, octopine dehydrogenase, phosphoenolpyruvate carboxykinase, cytosolic and mitochondrial glycerol 3-phosphate dehydrogenase and glutamate–oxaloacetate transaminase were measured in order to provide a qualitative indication of the importance of different processes for oxidation of glycolytically formed NADH. The muscles are divided into four groups: those that have a high activity of lactate dehydrogenase relative to the activities of phosphofructokinase (e.g. crustacean muscles); those that have high activities of octopine dehydrogenase but low activities of lactate dehydrogenase (e.g. scallop snap muscle); those that have moderate activities of both lactate dehydrogenase and octopine dehydrogenase (radular muscles of prosobranchs), and those that have low activities of both lactate dehydrogenase and octopine dehydrogenase, but which possess activities of phosphoenolpyruvate carboxykinase (oyster adductor muscles). It is suggested that, under anaerobic conditions, muscles of marine invertebrates form lactate and/or octopine or succinate (or similar end product) according to the activities of the enzymes present in the muscles (see above). The muscles investigated possess low activities of cytosolic glycerol 3-phosphate dehydrogenase, which indicates that glycerol phosphate formation is quantitatively unimportant under anaerobic conditions, and low activities of mitochondrial glycerol phosphate dehydrogenase, which indicates that the glycerol phosphate cycle is unimportant in the re-oxidation of glycolytically produced NADH in these muscles under aerobic conditions. Conversely, high activities of glutamate–oxaloacetate transaminase are present in some muscles, which indicates that the malate–aspartate cycle may be important in oxidation of glycolytically produced NADH under aerobic conditions. 3. High activities of nucleoside diphosphate kinase were found in muscles that function for prolonged periods under anaerobic conditions (e.g. oyster adductor muscles) and which possess phosphoenolpyruvate carboxylase activities. 4. In fast anaerobic muscles the activities of myofibrillar adenosine triphosphatase are about twice the calculated maximum rate of ATP formation from glycolysis. High activities of arginine kinase are found in these muscles. It is suggested that in these muscles arginine phosphate breakdown is quantitatively as important as carbohydrate degradation for the provision of ATP during anaerobic contraction.

It has been established that the maximum activities *in vitro* of certain enzymes in muscles from insects and vertebrates provide a reasonable quantitative assessment of the maximum flux through some metabolic pathways (Newsholme & Crabtree, 1973; for detailed discussion of theoretical and experimental bases of this approach see Crabtree & Newsholme, 1975). The results of such studies indicate the fuels oxidized by a particular muscle to support mechanical activity. In some cases, it is possible to provide an explanation for the biochemical nature of the fuel in relation to the physiological function of the muscle (see Crabtree & Newsholme, 1975).

Marine invertebrates are found in very different environments (e.g. pelagic, benthic, intertidal), which have necessitated the development of muscles with different physiological functions. For example, some muscles of the intertidal molluscs contract for long periods under anaerobic conditions, whereas the radular retractor muscles of prosobranch molluscs function for long periods under aerobic conditions. It was considered therefore that a comparative study of key enzymes of some metabolic pathways in muscles from marine invertebrates would provide quantitative information about energy metabolism in this group of animals.

The enzyme activities that have been measured are as follows: hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.11), which indicate the rate of glucose utilization; phosphorylase (EC 2.4.1.1) (and phosphofructokinase), which indicates the rate of glycogen degradation; phosphoenolpyruvate carboxykinase (EC 4.1.1.32), which indicates the proportion of the glycolytic flux that can be converted into succinate via the anaerobic reactions of the latter part of the tricarboxylic acid cycle (see Awapara & Simpson, 1967; Saz, 1971); and mitochondrial glycerol phosphate dehydrogenase (EC 1.1.99.5), which indicates the rate of the glycerol phosphate cycle (see Crabtree & Newsholme, 1972a). In addition, the activities of lactate dehydrogenase (EC 1.1.1.27), octopine dehydrogenase (EC 1.5.1.11) and cytoplasmic glycerol phosphate dehydrogenase (EC 1.1.1.8) have been measured, since they provide some qualitative indication of the biochemical processes for the re-oxidation of glycolytically produced NADH. The activities of arginine kinase (EC 2.7.3.3) have also been measured to compare, qualitatively, the importance of arginine phosphate with anaerobic glycolysis for energy formation in some muscles.

The anaerobic formation of succinate in marine invertebrates involves the phosphoenolpyruvate carboxykinase reaction which produces GTP (or ITP) rather than ATP. Since this reaction produces a third of the energy obtained from the succinate pathway and since the contractile process utilizes ATP rather than other nucleotides (see Needham, 1971), GTP must be converted into ATP. Con-

sequently the activities of the enzyme nucleoside diphosphate kinase (EC 2.7.4.6) have been measured in these muscles.

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for the following: mercaptoethanol, glycogen, arginine and all inorganic chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.; [^{14}C]arginine (270 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; Dowex exchange resin, tricaine (ethyl-7-aminobenzoate), Antimycin A (B grade) and bovine serum albumin (fatty acid-free) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.

Sources of animals

Most marine invertebrates were obtained from the University Marine Biology Station, Millport, Isle of Cumbrae, Scotland, U.K., or from the Marine Biological Association, Citadel Hill, Plymouth, Devon, U.K. Lobsters were obtained from Fisher Bros., 12 Billingsgate Street, London E.C.3, U.K. *Lepas antifer* and *Pachygrapsus marmoratus* were collected in Malta. Specimens of *Limulus polyphemus* were a gift from the Department of Zoology, University of Cambridge. Specimens of *Todarodes sagittatus* were caught in the N.E. Atlantic during a cruise on board R.R.S. Challenger.

Preparation of homogenates

Samples of *Metridium* basilar muscle were obtained by cutting the basal part of the column while the animals were attached to a smooth surface. The muscle obtained was washed with sea water and extraction medium (see below) to remove any contaminating non-muscle tissue. Polychaetes were anaesthetized with tricaine, which was dissolved in sea water (1:30000, w/v). Crustaceans were usually cooled on ice for about 15 min before dissection. Bivalves were cooled on ice and the adductor muscles of the gaping animals were cut to open the valves; the adductor muscles could then be dissected. In prosobranchs, the foot-retractor muscle was dissected after crushing the shell and exposing the soft parts of the animal. Radular muscles were obtained by dissecting out the muscle from the proboscis or buccal mass of the animals. Squids were killed by decapitation and mantle and dorsal parts of the fin muscle dissected immediately. Echinoids were cut in half horizontally, and the lantern retractor muscles dissected. *Thyone* pharyngeal retractors were obtained by irritating the animals either mechanically or by using tricaine in solution until the pharynx was

everted with the muscles attached. The muscles were then dissected off the pharyngeal structure.

Muscles were dissected as quickly as possible and were cut into small pieces before homogenization. Muscles were homogenized in ground-glass homogenizers with 5–20 vol. of extraction medium. The extraction medium for hexokinase, phosphoenolpyruvate carboxykinase, lactate dehydrogenase, octopine dehydrogenase, glutamate-oxaloacetate transaminase, nucleoside diphosphate kinase and cytoplasmic and mitochondrial glycerol 3-phosphate dehydrogenases contained 50 mM-triethanolamine, 1 mM-EDTA, 2 mM-MgCl₂, 30 mM-mercaptoethanol adjusted to pH 7.4 with KOH. The extraction medium for phosphofructokinase contained 70 mM-Tris/HCl, 1 mM-EDTA, 5 mM-MgSO₄ at pH 8.2 (Opie & Newsholme, 1967). For phosphorylase assays the extraction medium consisted of 35 mM-glycerol 2-phosphate, 20 mM-NaF, 1 mM-EDTA, 30 mM-mercaptoethanol at pH 6.2 (Cornblath *et al.*, 1963). For arginine phosphokinase assays the extraction medium consisted of 50 mM-Pipes [piperazine-*NN'*-bis-(2-ethanesulphonic acid)], 4 mM-dithiothreitol, 1 mM-EDTA at pH 7.0. The following enzymes were assayed after the homogenate was sonicated for two 15 s periods by using a MSE sonicator operating at an amplitude of 6 μ m: phosphoenolpyruvate carboxykinase, glutamate-oxaloacetate transaminase, nucleoside diphosphate kinase, mitochondrial glycerol phosphate dehydrogenase. The homogenate was cooled in ice/water during the sonication.

Assay of enzyme activities

Phosphofructokinase, hexokinase, lactate dehydrogenase, octopine dehydrogenase, cytoplasmic glycerol 3-phosphate dehydrogenase, glutamate-oxaloacetate transaminase, phosphoenolpyruvate carboxykinase and nucleoside diphosphate kinase were assayed by following the rate of change of E_{340} in a Gilford recording spectrophotometer (model 240) at 25°C.

Phosphofructokinase was assayed by the method described by Opie & Newsholme (1967), except that non-specific oxidation of NADH was inhibited by cyanide. The assay medium contained 50 mM-Tris/HCl, 6 mM-MgCl₂, 250 mM-KCl, 1 mM-ATP, 2 mM-AMP, 0.17 mM-NADH, 1 mM-KCN, 3 mM-fructose 6-phosphate, 4 μ g of glycerol 3-phosphate dehydrogenase (0.16 unit), 100 μ g of aldolase (0.9 unit) and 4 μ g of triose phosphate isomerase (9.6 units), to which 10–20 μ l of homogenate was added. The final volume in the cuvette was 2.0 ml. The final pH was 8.2. The assay was initiated by addition of fructose 6-phosphate. Controls, from which fructose 6-phosphate was omitted, were run concurrently.

Hexokinase was assayed in a medium containing 75 mM-Tris/HCl, 7.5 mM-MgCl₂, 0.8 mM-EDTA, 1.5 mM-KCl, 4 mM-mercaptoethanol, 0.4 mM-NADP⁺, 2.5 mM-ATP, 1 mM-glucose, 10 mM-creatine phos-

phate, 100 μ g of creatine kinase (1.8 units) and 10 μ g of glucose 6-phosphate dehydrogenase (1.4 units), to which was added 10–25 μ l of homogenate. The total volume was 2.0 ml and the final pH 7.3. The assay was initiated by the addition of glucose. Controls, from which glucose was omitted, were run concurrently.

Lactate dehydrogenase was assayed in a medium containing 50 mM-Tris/HCl, 0.17 mM-NADH, 0.3 mM- or 10 mM-sodium pyruvate and 1 mM-KCN, to which 10–20 μ l of homogenate was added. The final pH was 7.3. The total volume was 2.0 ml. The assay was initiated by the addition of pyruvate. Controls, from which pyruvate was omitted, were run concurrently.

Octopine dehydrogenase was assayed in a medium containing 50 mM-Tris/HCl, 0.17 mM-NADH, 1 mM-sodium pyruvate, 5 mM-DL-arginine and 1 mM-KCN, to which 10–20 μ l of homogenate was added. The total volume was 2.0 ml and the final pH was 7.0. The assay was initiated by the addition of arginine after a constant rate of change of E_{340} was obtained. Controls, from which arginine was omitted, were run concurrently.

Glutamate-oxaloacetate transaminase was assayed by the method of Bergmeyer & Bernt (1963). The assay medium contained 70 mM-potassium phosphate, 0.17 mM-NADH, 10 mM- α -oxoglutaric acid, 50 μ g of pyridoxal phosphate, 1 mM-KCN, 20, 25 or 70 mM-L-aspartate and 10 μ g of malate dehydrogenase (7.2 units) (in glycerol), to which was added 10 μ l of homogenate. The total volume was 2.0 ml and the final pH 7.5. The assay was initiated by the addition of aspartate. Controls, from which aspartate was omitted, were run concurrently. The V was calculated by extrapolation of double-reciprocal plots (activity against aspartate concentration) to infinite aspartate concentration.

Phosphoenolpyruvate carboxykinase was assayed by a method described by Opie & Newsholme (1967). The assay medium contained 80 mM-Tris/HCl, 0.17 mM-NADH, 1 mM-MnCl₂, 1 mM-MgCl₂, 1.5 mM-IDP, 1.1 mM-phosphoenolpyruvate, 20 mM-NaHCO₃, 5 μ g of Antimycin A and 10 μ g of malate dehydrogenase (7.2 units in glycerol), to which 10–20 μ l of homogenate was added. The total volume was 2.0 ml and the final pH 7.0. The assay was initiated by the addition of NaHCO₃. Controls, from which NaHCO₃ was omitted, were run concurrently; the rates of increase in E_{340} were less than 15% of the experimental rates.

Nucleoside diphosphate kinase was assayed by the method described by Parks & Agarwal (1973). The assay medium contained 90 mM-Tris/HCl, 25 mM-MgSO₄, 80 mM-KCl, 0.3 mM-NADH, 2 mM-ATP, 4 mM-phosphoenolpyruvate, 0.4 mM-dGDP, 2 μ g of pyruvate kinase (0.3 unit) and 5 μ g of lactate dehydrogenase (1.8 units), to which was added 5–10 μ l of homogenate. The total volume in the cuvette was

1.0ml and the final pH was 7.3. Controls, from which homogenate or dGDP was omitted, were run concurrently.

Cytoplasmic glycerol 3-phosphate dehydrogenase was assayed in a medium containing 70mM-Tris/HCl, 0.08mM-NADH, 0.4mM-dihydroxyacetone phosphate and 1mM-KCN, to which was added 10–20 μ l of homogenate. The total volume was 2.0ml and the final pH was 7.3. The assay was initiated by the addition of dihydroxyacetone phosphate. Controls, from which dihydroxyacetone phosphate was omitted, were run concurrently.

Mitochondrial glycerol 3-phosphate dehydrogenase was assayed by a modification of the method described by Pennington (1961) (see Crabtree & Newsholme, 1972a). The assay medium contained 50mM-KH₂PO₄, 1mM-KCN, 1.7mM-acetaldehyde, 20mM-DL-glycerol 3-phosphate, 200 μ g of yeast alcohol dehydrogenase and 0.05–0.08% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride, to which 10–100 μ l of homogenate was added. The final pH was 7.5 and the total volume 1.5ml, contained in stoppered glass tubes (10ml capacity). The assay was initiated by the addition of 100–250 μ l of aq. 0.5% (w/v) 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride. The reaction was terminated by the addition of 1.5ml of 10% (w/v) trichloroacetic acid, after periods of incubation of up to 30 min. Controls, from which glycerol 3-phosphate was omitted, were run concurrently for each time-interval and for each 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride concentration. The reduced dye was extracted in 4ml of ethyl acetate and the E_{490} of the ethyl acetate extract was measured by using a Zeiss (model M4Q III) spectrophotometer. The rate of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride reduction at infinite concentration of dye was calculated by extrapolation of double-reciprocal plots of the extinction versus the dye concentration. The molar extinction coefficient of reduced 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride in ethyl acetate was taken to be 20.1×10^3 litre \cdot mol⁻¹ \cdot cm⁻¹ (Pennington, 1961). The rates obtained were multiplied by a factor of 2.5 (see Crabtree & Newsholme, 1972a) in order to obtain maximum enzyme activities under these conditions.

Arginine kinase was assayed radiochemically by the method of Leech *et al.* (1976). The assay medium contained 75mM-Pipes, 10mM-ATP, 15mM-L-arginine, 2mM-dithiothreitol, 20mM-magnesium acetate, 5mM-phosphoenolpyruvate and 0.05 μ Ci (about 0.02nmol) of [U-¹⁴C]arginine, to which was added 10 μ l of homogenate. The final pH was 7.0 and the total volume was 10 μ l contained in small plastic tubes. The assay was initiated by the addition of homogenate and the stoppered tubes were incubated at 25°C for periods up to 20min. The reaction was terminated by the addition of 50 μ l of ethanolic solu-

tion of 20mM-HgCl₂. Portions (0.1 ml) of the incubation mixture were then transferred on to small columns (about 5cm in length constructed from Pasteur pipettes) of Dowex X-50 (Na⁺ form) ion-exchange resin in order to separate the [¹⁴C]arginine phosphate formed during the incubation. The columns were eluted, with 1.0ml of water, into scintillation vials. The eluate was evaporated to dryness and the residue was dissolved in 0.1ml of 1M-HCl; 1vol. of scintillant (ethoxyethanol/toluene, 3:5, v/v; see Hall & Cocking, 1965) was then added and the radioactivity of the [¹⁴C]arginine phosphate was measured in a Beckman liquid-scintillation system (model LS 230).

Phosphorylase was assayed by a modification of the method described by Cornblath *et al.* (1963) (see Crabtree & Newsholme, 1972a) in which the P_i liberated from glucose 1-phosphate during the synthesis of glycogen is measured. The assay medium contained 16mM-glucose 1-phosphate, 0.25mM-AMP and 1% (w/v) oyster glycogen, to which was added 0.2ml of homogenate. The total volume was 0.4ml, contained in small plastic tubes which were incubated at 25°C for periods up to 15 min. The assay was initiated by the addition of homogenate and terminated by the addition of 0.6ml of 6% (w/v) HClO₄. P_i was measured by the method of Atkinson *et al.* (1973). Controls, including water instead of glucose 1-phosphate and AMP, were run for each time-interval. Other controls, which included glycerol 3-phosphate instead of glucose 1-phosphate, were run in order to obtain rates for non-specific phosphatase activity.

Myofibrillar ATPase (adenosine triphosphatase) was assayed by following the rate of H⁺ release that accompanies hydrolysis of ATP by using a pH-stat with an automatic titrator (SBR-A Titrigraph and TTT-1 titrator; Radiometer, Copenhagen, Denmark). This method allowed the measurement of ATPase activities in the presence of phosphate (see Jones *et al.*, 1970). The assay medium contained 30mM-KCl (10mM-KCl for myofibrils from scallop adductor muscles), 1mM-MgCl₂, 0.01mM-CaCl₂ and 0.5mM-ATP, to which 0.5ml of myofibril suspension was added. The total volume was 10.0ml and the final pH was 7.5. The assay was initiated by the addition of ATP. Controls from which CaCl₂ was omitted, but which contained 0.1mM-EGTA [ethanedioxybis-(ethylamine)tetra-acetate], were run concurrently.

Results and Discussion

Control experiments on conditions of enzyme assay

The aim of this comparative study is to provide reliable information on the maximum activities of certain key enzymes from various muscles from a number of marine invertebrates. One difficulty in such an analysis is the possibility of variation in the proper-

ties of the enzyme from one animal to another. To overcome such difficulties totally, a detailed analysis of the properties of the enzymes from each animal should be carried out. However, the amount of work involved would restrict the accumulation of data to a very few animals. In the present work, the effect of temperature, pH and substrate concentration have been investigated with the enzymes from muscles of selected animals representing the major phyla investigated. In this control study the muscles investigated were as follows: basilar muscle of the sea-anemone, longitudinal muscle of the ragworm, adductor muscles of the sand gaper, the edible oyster and the scallop, pedal retractor muscle of the winkle, claw adductor muscle of the edible crab, the abdominal muscle of the lobster, the radular retractor muscle of the limpet and the fin muscle of the common squid.

It was considered that the activities should be measured at 25°C, since they would be directly comparable with those of other studies (e.g. Crabtree & Newsholme, 1972*a,b*; Alp *et al.*, 1976). This temperature may be greater than the normal for marine invertebrates, although it should be pointed out that the sea temperature may be as high as 21–22°C in the summer and the body temperature of marine invertebrates may be higher than this if they are present in pools on the shore at low tide. Nonetheless, the activities of several enzymes was measured at both 10° and 25°C. The results are reported in Table 1. The increases in activities for the various enzymes from the different muscles over this temperature range were similar (approx. threefold). A similar effect of temperature was observed in this work on the activities of citrate synthase, which suggests that activities reported in the present work can be compared with those of the tricarboxylic acid-cycle enzymes reported by Alp *et al.* (1976). These results suggest that there are no unusual effects of temperature on these enzymes between 10° and 25°C, so that comparison of activities at 25°C is satisfactory.

The pH optima were determined for the enzymes phosphofructokinase, phosphoenolpyruvate carboxykinase, lactate dehydrogenase and octopine dehydrogenase. In general, the pH optimum for any particular enzyme lay within 0.2pH unit of the pH used for the assay of enzyme activities for comparative studies (see the Materials and Methods section). For the animals investigated the difference between the activity at the pH optimum and that at the pH used experimentally was never greater than 5%.

The responses of several enzymes investigated, including phosphofructokinase, glycerol 3-phosphate dehydrogenase, phosphoenolpyruvate carboxykinase, arginine kinase, lactate dehydrogenase and octopine dehydrogenase, to substrate and cofactor concentrations have been studied in detail for muscles from many animals, including marine invertebrates (for details see Crabtree & Newsholme, 1972*a,b*;

Table 1. Activities of phosphofructokinase, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate carboxykinase and citrate synthase in muscles from various animals at 10° and 25°C

Animal	Muscle	Enzyme activities (μmol/min per g fresh wt. of muscle)									
		Phosphofructokinase		Lactate dehydrogenase		Pyruvate kinase		Phosphoenolpyruvate carboxykinase		Citrate synthase	
		10°C	25°C	10°C	25°C	10°C	25°C	10°C	25°C	10°C	25°C
Sand gaper (<i>Mya arenaria</i>)	Adductor	0.9	2.7	—	—	2.1	6.7	0.4	1.3	2.8	6.9
Great scallop (<i>Pecten maximus</i>)	Striated adductor	2.1	7.4	—	—	17.0	54.0	—	—	—	—
Common oyster (<i>Osirea edulis</i>)	Phasic adductor	0.6	2.6	—	—	1.2	4.2	0.3	1.0	—	—
Thick top shell (<i>Monodonta lineata</i>)	Pedal retractor	1.3	5.1	—	—	8.2	32.0	0.5	1.3	—	—
Edible crab (<i>Cancer pagurus</i>)	Claw adductor	2.5	9.6	2.1	6.0	11.7	56.0	—	—	1.4	2.4
Ragworm (<i>Nereis virens</i>)	Longitudinal	2.7	8.5	5.3	11.0	21.5	75.0	—	—	2.8	6.5

Enzyme activities were measured as described in the Materials and Methods section. Activities are presented as means of measurements on extracts from three different animals and the variation about the mean was less than 20%.

Leech, 1973; Zammit, 1974; Sugden & Newsholme, 1975; Zammit & Newsholme, 1976). The results of these studies indicate that the concentrations of substrates and cofactors used in the present work are sufficient to provide maximum activities of the enzymes under investigation.

Maximum activities of hexokinase, phosphorylase and phosphofructokinase in relation to the calculated maximum rates of anaerobic glycolysis

Crabtree & Newsholme (1972a) used oxygen- and glucose-uptake data to calculate maximum glycolytic fluxes in specific muscles during mechanical activity *in vivo*. However, this information is not available for marine invertebrate muscles. If the major energy-requiring process is the contractile system in these muscles, the maximum rate of ATP utilization *in vivo* could be estimated from the maximum ATPase activity of the extracted muscle *in vitro*. From these data, the maximum possible flux through the energy-producing pathways could be calculated (assuming knowledge of the number of ATP molecules generated in the pathways). Consequently, it was decided to measure the maximum activity of myofibrillar ATP-

ase in selected muscles, from which the maximum rate of anaerobic glycolysis required to support this activity could be calculated (see legend to Table 2). (This ATPase activity represents an underestimate of the maximum rate of ATP utilization *in situ*, owing to the presence of other ATP-utilizing systems.) The latter data are presented together with some of the enzyme activities *in vitro* in Table 2. The calculated rates of glucose utilization are considerably greater than hexokinase activities in all muscles investigated except the radular retractor of the limpet. However, there is reasonable agreement between the activities of phosphofructokinase and the maximum calculated rate of anaerobic glycolysis, which indicates that glycogen rather than glucose is the important carbohydrate fuel in these muscles. This extent of agreement supports the view that phosphofructokinase activity provides a reasonably quantitative indication of glycolytic flux (see Crabtree & Newsholme, 1972a). However, in a number of muscles the activity of this enzyme is considerably less than the flux through glycolysis, calculated on the basis of energy requirements by these muscles (phasic adductors of the scallop and oyster; pedal retractor of the periwinkle;

Table 2. Activities of hexokinase, phosphofructokinase and myofibrillar ATPase and the calculated rates of carbohydrate utilization in selected muscles of some marine invertebrates

The enzyme activities were measured as described in the Materials and Methods section. The range of activities for the glycolytic enzymes is given in Table 3. The ranges of activities of myofibrillar ATPase (on the basis of mg of myofibrillar protein) are not given, but they were not greater than 20% of the mean. The rate of carbohydrate utilization is calculated by assuming that ATP is produced anaerobically from the conversion of glycogen into lactate (i.e. 3 ATP molecules/glucose residue) or from the conversion of glycogen into succinate (i.e. 4 ATP molecules/glucose residue) for the oyster (see below). Thus for all the animals, except the oyster, the myofibrillar ATPase activity is divided by 3 to produce an estimate of glycolytic flux. For the oyster, the ATPase activity is divided by 4.

Animal	Muscle	Rate of carbohydrate utilization calculated from myofibrillar ATPase activity ($\mu\text{mol}/\text{min}$ per g fresh wt. at 25°C)	Enzyme activities ($\mu\text{mol}/\text{min}$ per g fresh wt. at 25°C)		
			Myofibrillar ATPase	Hexokinase	Phosphofructokinase
Great scallop (<i>Pecten maximus</i>)	Phasic adductor	23.7	71.1	<0.1	9.4
	Catch adductor	1.9	5.8	0.2	4.5
Common oyster (<i>Ostrea edulis</i>)	Phasic adductor	12.4	49.6	0.6	2.8
Razor clam (<i>Ensis ensis</i>)	Pedal retractor	17.8	53.3	0.5	6.5
Periwinkle (<i>Littorina littorea</i>)	Pedal retractor	3.5	10.5	0.8	12.4
Thick top shell (<i>Monodonta lineata</i>)	Pedal retractor	10.0	30.0	1.0	10.9
Common limpet (<i>Patella vulgata</i>)	Radular retractor	3.9	11.6	3.1	13.3
Common squid (<i>Loligo forbesi</i>)	Fin	11.4	34.1	0.4	10.5
	Mantle	20.8	61.5	0.1	9.8
Squat lobster (<i>Galathea squamifera</i>)	Abdominal flexor	10.2	30.7	0.3	6.5
Lobster (<i>Homarus vulgaris</i>)	Abdominal flexor	19.7	59.1	0.6	9.9
	Claw adductor	9.2	27.7	0.6	6.0

Table 3. Maximum activities of hexokinase, phosphorylase and phosphofructokinase in muscles from marine invertebrates

Enzyme activities were measured as described in the Materials and Methods section, and they are presented as means, with the ranges and the numbers of separate animals used in parentheses.

Animal	Muscle	Enzyme activities ($\mu\text{mol/min}$ per g fresh wt. of muscle at 25°C)		
		Hexokinase	Phosphorylase	Phosphofructokinase
Coelenterata (Anthozoa)				
Sea-anemone (<i>Metridium senile</i>)	Basilar	1.6 (1.5, 1.7) (2)	0.5 (0.5, 0.5) (2)	1.2 (0.5–2.0) (4)
	Sphincter	1.0 (0.7–1.3) (3)	0.5 (0.5, 0.5) (2)	0.9 (0.7–1.0) (3)
Annelida (Polychaeta)				
Sea-mouse (<i>Aphrodite aculeata</i>)	Longitudinal	0.4 (0.3–0.5) (3)	4.0 (3.7, 4.3) (2)	8.6 (7.8–9.2) (3)
Ragworm (<i>Nereis virens</i>)	Dorsal longitudinal	0.7 (0.7, 0.7) (2)	11.5 (11.4, 11.6) (2)	10.3 (8.4–11.5) (5)
Mollusca (Bivalvia)				
Great scallop (<i>Pecten maximus</i>)	Phasic adductor	<0.1 (2)	8.5 (6.3–10.6) (3)	9.4 (7.5–13.8) (4)
	Catch adductor	0.2 (0.1, 0.2) (2)	1.8 (1.5–2.3) (3)	4.5 (3.1–6.0) (6)
Variegated scallop (<i>Chlamys varius</i>)	Phasic adductor	0.1 (0.1, 0.1) (2)	1.9 (1.6, 2.1) (2)	3.2 (2.5–3.8) (5)
	Catch adductor	0.5 (0.5, 0.5) (2)	—	3.8 (3.1–4.8) (4)
Horse mussel (<i>Modiolus modiolus</i>)	Phasic adductor	1.0 (0.8, 1.1) (2)	1.0 (0.9, 1.1) (2)	4.4 (3.4–5.2) (4)
	Catch adductor	0.4 (0.4, 0.4) (2)	0.5 (0.3, 0.7) (2)	2.9 (2.1–3.8) (3)
Striped venus (<i>Venus striatula</i>)	Phasic adductor	1.6 (1.1, 2.1) (2)	—	7.3 (6.6–8.2) (3)
Common oyster (<i>Ostrea edulis</i>)	Phasic adductor	0.6 (0.4–0.7) (3)	1.4 (0.8–2.1) (3)	2.8 (2.0–3.6) (3)
	Catch adductor	0.2 (0.2, 0.2) (2)	0.3 (0.2, 0.4) (2)	0.8 (0.8, 0.8) (2)
Sand gaper (<i>Mya arenaria</i>)	Adductor	1.6 (1.0–2.6) (3)	1.8 (1.1–2.1) (5)	2.6 (2.5–2.7) (3)
Razor clam (<i>Ensis ensis</i>)	Pedal retractor	0.5 (0.4, 0.5) (2)	4.8 (4.1–6.0) (3)	6.5 (5.1–9.1) (3)
(Gastropoda)				
Common top shell (<i>Mondonta turbinata</i>)	Pedal retractor	0.5 (0.4–0.5) (3)	10.9 (10.3, 11.5) (2)	5.2 (4.5–6.9) (4)
Periwinkle (<i>Littorina littorea</i>)	Pedal retractor	0.8 (0.6–1.1) (4)	7.8 (7.1, 8.2) (2)	12.4 (11.0–18.9) (5)
Thick top shell (<i>Monodonta lineata</i>)	Pedal retractor	1.0 (0.6–1.8) (3)	3.8 (3.2–4.2) (3)	10.9 (10.3–11.4) (3)
Common whelk (<i>Buccinum undatum</i>)	Radular retractor	3.7 (2.1–5.8) (5)	21.6 (17.7–23.4) (4)	19.6 (17.9–21.9) (4)
Common limpet (<i>Patella vulgata</i>)	Radular retractor	3.1 (2.7–3.5) (3)	3.0 (2.7–3.3) (2)	13.3 (10.1–17.2) (5)
Winkle (<i>Murex trunculus</i>)	Radular retractor	15.5 (11.3–21.0) (5)	12.0 (8.8–16.9) (3)	23.8 (21.8–26.7) (3)
(Cephalopoda)				
Oceanic squid (<i>Toradodes sagittatus</i>)	Fin	2.8 (2.3–3.3) (3)	9.9 (9.0–12.6) (4)	9.4 (8.4–10.8) (3)
Common squid (<i>Loligo forbesi</i>)	Fin	0.4 (0.3–0.5) (3)	10.8 (9.5–11.7) (3)	10.5 (8.1–13.7) (3)
	Mantle	0.1 (0.1, 0.1) (2)	10.9 (10.2–11.7) (3)	9.8 (7.6–14.2) (4)

Table 3—continued

Animal	Muscle	Enzyme activities ($\mu\text{mol/min per g fresh wt. of muscle at } 25^\circ\text{C}$)		
		Hexokinase	Phosphorylase	Phosphofructokinase
Arthropoda (Crustacea)				
Lobster (<i>Homarus vulgaris</i>)	Deep abdominal flexor	0.6 (0.3–1.4) (6)	8.6 (8.2–8.7) (3)	9.9 (7.0–13.8) (4)
	Claw adductor	0.6 (0.3–1.3) (4)	4.4 (4.3, 4.5) (2)	6.0 (4.6–8.0) (4)
Squat lobster (<i>Galathea squamifera</i>)	Abdominal flexor	0.3 (0.2–0.4) (3)	16.0 (11.9–20.1) (3)	6.5 (4.0–11.6) (5)
Shrimp (<i>Crangon allmanni</i>)	Abdominal flexor	0.1 (0.1) (3)	6.5 (5.8–7.0) (3)	7.4 (6.5–8.4) (3)
Mediterranean crab (<i>Pachygrapsus marmoratus</i>)	Leg	0.9 (0.5–1.4) (3)	13.2 (12.5, 13.9) (2)	9.0 (6.8–12.5) (3)
Swimming crab (<i>Portunus puber</i>)	Leg	1.2 (0.9–1.4) (5)	12.6 (9.8–15.3) (3)	15.0 (11.5–20.5) (3)
Edible crab (<i>Cancer pagurus</i>)	Claw adductor	0.8 (0.7–1.0) (3)	8.9 (8.9, 8.9) (2)	9.6 (7.2–13.6) (5)
	Leg	1.0 (1.0, 1.0) (2)	—	14.7 (13.0–16.4) (3)
Pedunculate barnacle (<i>Lepas anatifera</i>)	Closer	2.2 (1.7–3.3) (4)	1.9 (1.7, 2.1) (2)	5.4 (5.0–6.0) (3)
(Chelicerata)				
Horse-shoe crab (<i>Limulus polyphemus</i>)	Leg	1.4 (0.9–1.8) (3)	—	13.8 (11.2–17.2) (3)
Echinodermata (Echinoidea)				
Common sea-urchin (<i>Echinus esculentus</i>)	Lantern retractor	1.6 (1.4–1.8) (3)	1.2 (1.2, 1.2) (2)	1.4 (1.2–1.8) (3)
(Holothuroidea)				
Sea-cucumber (<i>Thyone</i> sp.)	Pharyngeal retractor	0.6 (0.3–0.7) (4)	—	0.5 (0.4–0.9) (4)

mantle of the squid; abdominal flexor of the lobster). This suggests that another process(es) must supplement glycolysis for energy production in these muscles (e.g. breakdown of arginine phosphate; see below).

Activities of hexokinase, phosphorylase and phosphofructokinase in relation to the function of the muscles

For the 28 species investigated, the activities of the enzymes range from 0.1 to 15.5, 0.3 to 21.6 and 0.5 to 23.8 $\mu\text{mol/min per g fresh wt.}$ for hexokinase, phosphorylase and phosphofructokinase respectively (Table 3). In most muscles, the activities of phosphofructokinase are similar to those of phosphorylase. The results indicate that the muscles can be classified into three groups according to the activities of hexokinase in comparison with the activities of the other two enzymes.

(1) *Low activities of all three enzymes.* Low activities of the enzymes are found in coelenterate muscle, in catch muscles of the molluscs and in the muscles of the echinoderms (Table 3). These findings suggest a low rate of energy utilization by the contractile process in these muscles. From another type of study, it is

known that a low rate of energy utilization is required for maintaining closure of the shells in the catch muscles (Rüegg, 1971).

(2) *High activities of phosphorylase plus phosphofructokinase and low activities of hexokinase.* High activities of phosphorylase and phosphofructokinase but low activities of hexokinase are found in muscles that perform short but rapid bursts of contraction (e.g. longitudinal muscles of polychaetes, striated adductors of bivalve molluscs, mantle muscle of squid, abdominal flexors of decapod crustaceans; see Table 3). In general, these muscles have a low capacity of the tricarboxylic acid cycle (as indicated by maximum activities of some of the enzymes of the cycle; see Alp *et al.*, 1976). It is suggested that they depend largely on the anaerobic degradation of glycogen for energy production, so that they are similar to the anaerobic vertebrate muscles (Crabtree & Newsholme, 1972a). However, the end products of anaerobic metabolism may be different in some of these invertebrate muscles (see below).

Slightly higher activities of hexokinase and the tricarboxylic acid-cycle enzymes are present in the leg muscles of the swimming crab and horse-shoe crab

(see Table 3 and Alp *et al.*, 1976). This suggests that oxidation of glucose may provide energy for the more prolonged activity of these muscles that would be involved in the locomotion of the animals.

(3) *High activities of hexokinase.* The radular muscles of the prosobranchs and the fin muscles of the squid contain higher activities of hexokinase than do muscles of other animals investigated (Table 3). This finding is consistent with the higher activities of the enzymes of the tricarboxylic acid cycle in these muscles (see Alp *et al.*, 1976). In the fin muscles of the squids, *Todarodes* and *Loligo*, the activities of citrate synthase are 25.9 and 15.5, and the activities of NAD⁺- and NADP⁺-linked isocitrate dehydrogenases are 0.3 and 31.0 and 0.2 and 29.6 $\mu\text{mol}/\text{min per g}$ respectively. Both fin muscles of the squid and the radular retractor muscles are known to be mechanically active for long periods of time (the radular muscles in the limpets are involved in feeding, and this may proceed continually for 24 h under experimental conditions; A. J. Southward, personal communication). Since the energy requirements for maintenance of such sustained mechanical activity are large, blood-borne fuels, which are usually available in greater quantities than endogenous fuels, must be completely oxidized. This is consistent with the high activities of hexokinase and the tricarboxylic acid-cycle enzymes in these muscles.

Oxidation of glycolytically produced NADH

The glyceraldehyde 3-phosphate dehydrogenase reaction reduces NAD⁺ to NADH, and to maintain the rate of glycolysis the NADH must be reoxidized. Since carbohydrate utilization is quantitatively important in energy provision for muscles of marine invertebrates (see above), the oxidation of NADH must also be important. Consequently, the activities of lactate dehydrogenase, octopine dehydrogenase, cytoplasmic and mitochondrial glycerol phosphate dehydrogenase and glutamate-oxaloacetate transaminase have been measured in these muscles to provide qualitative information on the processes used for NADH re-oxidation. Since these enzymes (except mitochondrial glycerol 3-phosphate dehydrogenase) catalyse near-equilibrium reactions in the cell, their activities cannot provide quantitative information (see Crabtree & Newsholme, 1975). The malate-aspartate cycle (see Chappell, 1968) and the glycerol phosphate cycle (Sacktor, 1965) have been proposed as shuttles to transfer hydrogen ions into the mitochondria for oxidation under aerobic conditions. The maximum flux through the latter cycle is estimated from the maximum activities of the mitochondrial glycerol phosphate dehydrogenase (Crabtree & Newsholme, 1972a). In the present work the activity of this enzyme was very low (in comparison with the maximum glycolytic flux) in all muscles investigated (<0.7 $\mu\text{mol}/\text{min per g}$; see Table 4), including the

aerobic radular retractor muscles of the prosobranch molluscs and the fin muscles of the squids (see Alp *et al.*, 1976). In general, muscles of the marine invertebrates utilize carbohydrate mainly under anaerobic conditions, so that low activities of the enzymes of the glycerol phosphate cycle are expected. However, the radular retractor muscles and the fin muscles are considered to be aerobic and possess activities of the enzymes of the tricarboxylic acid cycle that approach those present in some insect flight muscles (see Alp *et al.*, 1976). It is well established that insect flight muscles oxidize most, if not all, of the glycolytically produced NADH via the glycerol phosphate cycle, but this is not the case for other invertebrate or vertebrate muscles (see above and Crabtree & Newsholme, 1972a). The present work suggests that insect flight muscles are unique in dependence on a glycerol phosphate cycle for aerobic NADH oxidation. This suggestion contrasts with that of Storey & Hochachka (1975), who consider that squid muscle has a significant activity of the glycerol phosphate cycle.

The activities of glutamate-oxaloacetate transaminase are high in the fin muscles of the squid, the radular muscles of the prosobranchs and the leg muscle of the swimming crab (43.7, 50.2 and 291 $\mu\text{mol}/\text{min per g}$ in the fin muscle of the squid, leg muscle of the swimming crab and the radular retractor muscle of the whelk respectively; see Table 4). This suggests that these muscles utilize the malate-aspartate cycle for oxidation of glycolytically produced NADH under aerobic conditions.

Under anaerobic conditions the formation of lactate, the formation of octopine or the formation of succinate (or similar intermediates) are important in the re-oxidation of NADH in muscles of marine invertebrates (Awapara & Simpson, 1967; Robin & Thoi, 1961; Hochachka & Mustafa, 1972). The present work indicates in which muscles of the marine invertebrates these processes may be important. Low activities of lactate dehydrogenase were found in all muscles of coelenterates, molluscs (except for the radular muscles of the whelk) and the lantern retractor muscles of *Echinus esculentus* (<0.1–6.4 $\mu\text{mol}/\text{min per g}$ for many muscles and radular retractor of the winkle; see Table 4). However, high activities of lactate dehydrogenase (in comparison with the maximum rates of glycolysis) are present in the crustacean muscles and the pharyngeal retractor muscles of *Thyone* sp. (8.7–127.2 $\mu\text{mol}/\text{min per g}$ for claw adductor muscle of the edible crab and abdominal flexor of the lobster respectively). In these latter muscles, the lactate dehydrogenase reaction appears to be important in the re-oxidation of NADH, since the activities of this enzyme are high, whereas those of octopine dehydrogenase are low. It is interesting that, in at least some of these muscles, the lactate dehydrogenase isoenzyme activity ratio is similar to that from

Table 4. Activities of lactate dehydrogenase, octopine dehydrogenase, cytoplasmic glycerol 3-phosphate dehydrogenase, mitochondrial glycerol-3-phosphate dehydrogenase and glutamate-oxaloacetate transaminase in muscles of marine invertebrates

The enzyme activities were measured as described in the Materials and Methods section and they are given as means; the ranges and the numbers of separate animals used are given in parentheses. For common names see Table 3.

Animal	Muscle	Enzyme activities ($\mu\text{mol}/\text{min}$ per g fresh wt. of muscle at 25°C)					
		Lactate dehydrogenase		Octopine dehydrogenase	Glycerol 3-phosphate dehydrogenase		Glutamate-oxaloacetate transaminase
		0.3 mM-Pyruvate	10 mM-Pyruvate		Cytoplasmic	Mitochondrial	
Coelenterata (Anthozoa) <i>Metridium senile</i>	Basilar	<0.1 (3)	<0.1 (3)	7.1 (6.6-7.9) (3)	0.2 (0.2, 0.2) (2)	—	—
	Sphincter	<0.1 (3)	<0.1 (3)	—	—	—	—
Annelida (Polychaeta) <i>Aphrodite aculeata</i>	Longitudinal	0.5 (0.4, 0.7) (2)	0.4 (0.2, 0.5) (2)	<0.1 (2)	—	<0.1 (2)	5.6 (4.2-7.1) (3)
	Dorsal longitudinal	11.6 (11.0-12.5) (3)	17.6 (15.5-21.1) (3)	<0.1 (3)	0.3 (0.3, 0.3) (2)	—	—
Mollusca (Bivalvia) <i>Pecten maximus</i>	Phasic adductor	<0.1 (3)	<0.1 (3)	29.5 (28.8-30.5) (3)	—	<0.1 (2)	24.5 (21.0-28.0) (3)
	Catch adductor	<0.1 (3)	<0.1 (3)	15.5 (15.1, 15.9) (2)	—	<0.1 (2)	7.4 (5.2-9.0) (4)
<i>Chlamys varius</i>	Phasic adductor	<0.1 (2)	<0.1 (2)	30.2 (25.6-32.7) (3)	—	0.5 (0.5) (2)	17.4 (16.7-18.7) (3)
	Catch adductor	<0.1 (2)	<0.1 (2)	—	—	<0.1 (2)	14.3 (11.1-19.5) (4)
<i>Modiolus modiolus</i>	Phasic adductor	0.3 (0.3, 0.3) (2)	0.7 (0.7, 0.7) (2)	—	0.1 (2)	—	5.4 (5.2-6.0) (3)
	Catch adductor	0.4 (0.4, 0.4) (2)	0.5 (0.5, 0.5) (2)	—	—	—	3.7 (2.9-4.3) (3)
<i>Glycymeris glycymeris</i>	Phasic adductor	0.1 (0.1, 0.1) (2)	0.4 (0.4, 0.4) (2)	—	0.6 (0.5-0.8) (3)	—	5.2 (4.9-5.3) (3)
	Phasic adductor	0.4 (0.3-0.5) (4)	1.2 (0.5-2.0) (4)	—	0.4 (0.4, 0.4) (2)	—	16.8 (15.1, 18.5) (2)
<i>Venus striatula</i>	Phasic adductor	0.1 (0.1, 0.1) (2)	0.2 (0.2, 0.2) (2)	<0.1 (2)	0.8 (0.8, 0.8) (2)	<0.1 (2)	8.6 (7.5-9.5) (3)
	Catch adductor	0.2 (0.2, 0.2) (2)	0.4 (0.3, 0.4) (2)	<0.1 (2)	—	—	5.5 (4.5-6.3) (4)
<i>Ostrea edulis</i>	Phasic adductor	0.1 (0.1, 0.1) (2)	0.2 (0.2, 0.2) (2)	<0.1 (2)	—	—	—
	Catch adductor	0.2 (0.2, 0.2) (2)	0.4 (0.3, 0.4) (2)	<0.1 (2)	—	—	—
<i>Ensis ensis</i>	Phasic adductor	<0.1 (3)	<0.1 (3)	62.4 (56.2-67.3) (4)	—	—	—
	Pedal retractor	<0.1 (3)	<0.1 (3)	—	—	—	—

(Gastropoda)	Pedal retractor	<0.1	(3)	<0.1	<0.1	(3)	—	—	—
<i>Monodonta turbinata</i>									
<i>Littorina littorea</i>	Pedal retractor	2.4	(3)	4.8	(4, 7, 4.9)	(2)	1.9	(1.7, 2.0)	(2)
<i>Monodonta lineata</i>	Pedal retractor	0.2	(4)	0.4	(0.4–0.5)	(3)	<0.1	<0.1	(2)
<i>Buccinum undatum</i>	Radular retractor	4.9	(3)	23.3	(20.1–32.8)	(4)	22.4	2.8	0.7
<i>Patella vulgata</i>	Radular retractor	0.6	(3)	1.4	(1.1–1.8)	(3)	17.7	0.2	0.5
<i>Murex trunculus</i>	Radular retractor	1.8	(5)	6.4	(5.7–6.8)	(5)	—	—	—
(Cephalopoda)									
<i>Todarodes sagittatus</i>	Fin	<0.1	(2)	<0.1	(30.0–46.0)	(4)	38.5	12.7	0.7
<i>Loligo forbesi</i>	Fin	<0.1	(2)	<0.1	(85.0–145.1)	(4)	113.0	16.7	0.5
	Mantle	<0.1	(2)	<0.1	(143.0–151.0)	(3)	146.5	10.8	0.5
Arthropoda (Crustacea)									
<i>Homarus vulgaris</i>	Deep abdominal flexors	127	(5)	56.0	(10.1–14.6)	(4)	<0.1	0.5	0.5
	Claw adductor	10.3	(3)	34.3	(15.1–18.0)	(3)	<0.1	—	—
<i>Galathea squamifera</i>	Abdominal flexor	45.1	(2)	20.7	(8.7, 12.8)	(2)	<0.1	—	—
<i>Crangon allmanni</i>	Abdominal flexor	22.4	(3)	9.6	(0.5, 0.5)	(2)	<0.1	—	—
<i>Pachygrapsus marmoratus</i>	Leg	21.2	(3)	75.2	(0.5, 0.5)	(2)	<0.1	—	—
<i>Portunus puber</i>	Leg	51.0	(3)	24.3	(0.5, 0.6)	(2)	<0.1	0.6	50.2
<i>Cancer pagurus</i>	Claw adductor	8.7	(2)	35.4	(0.3–0.5)	(3)	<0.1	—	—
	Leg	19.4	(2)	77.0	(0.3–0.5)	(3)	<0.1	0.4	—
(Chelicerata)									
<i>Limulus polyphemus</i>	Leg	120	(2)	35.0	(30.0, 39.0)	(2)	<0.1	—	—
Echinodermata (Echinoidea)									
<i>Echinus esculentus</i>	Lantern retractor	2.5	(3)	2.4	(2.2–2.6)	(3)	<0.1	—	—
(Holothuroidea)									
<i>Thyone</i> sp.	Pharyngeal retractor	18.0	(3)	48.1	(41.1–53.5)	(3)	<0.1	—	—

the white vertebrate muscles (see Table 4 and Crabtree & Newsholme, 1972a).

High activities of octopine dehydrogenase (in comparison with the maximum rates of glycolysis) but low activities of lactate dehydrogenase were found in the basilar muscle of the sea-anemone, the pedal retractor of the razor clam, the fin and mantle muscles of the cephalopods and the adductor muscles of scallops (but not any of the other bivalves) (7.1–146.5 $\mu\text{mol/min per g}$ for the basilar muscle of the sea-anemone and the mantle muscle of the squid respectively; see Table 4). Consequently, the conversion of pyruvate and arginine into octopine is probably the main process whereby NADH is re-oxidized in these muscles. The radular retractor muscle of the whelk contains similar activities of lactate dehydrogenase and octopine dehydrogenase (about 22 $\mu\text{mol/min per g}$; see Table 4). However, the combined activities of these two enzymes are not greater than the maximum glycolytic flux. This suggests that these muscles may not be mechanically active under anaerobic conditions.

Some molluscan muscles (e.g. oyster) contain very low activities of both lactate dehydrogenase and

octopine dehydrogenase and a low aerobic capacity (see Table 4 and Alp *et al.*, 1976). This suggests that these muscles re-oxidize NADH under anaerobic conditions via reactions that lead to the formation of succinate (or other anaerobic end products that involve reactions of the tricarboxylic acid cycle; see below and Awapara & Simpson, 1967; Stokes & Awapara, 1968; de Zwaan & von Marrewijk, 1973).

Activities of phosphoenolpyruvate carboxykinase and nucleoside diphosphate kinase

The conversion of glucose into succinate in the adductor muscles of some marine bivalves increases the yield of ATP compared with the formation of lactate (see Sanadi & Fluharty, 1963). This is of importance in these muscles, since the duration of anaerobiosis depends on tidal conditions, so that it may be prolonged for several hours. Some indication of the significance of the succinate pathway for energy production in marine invertebrates is obtained from a comparison of the activities of phosphoenolpyruvate carboxylase in these different muscles (see Table 5). Activities of about 1 $\mu\text{mol/min per g}$ are found in the

Table 5. *Activities of phosphoenolpyruvate carboxykinase and nucleoside diphosphate kinase in muscle of marine invertebrates*

Enzyme activities were measured as described in the Materials and Methods section and they are presented as means, with the ranges and the numbers of animals used in parentheses. Common names are given in Table 3.

Animal	Muscle	Enzyme activities ($\mu\text{mol/min per g}$ fresh wt. of muscle at 25°C)	
		Phosphoenolpyruvate carboxykinase	Nucleoside diphosphate kinase
Coelenterata (Anthozoa) <i>Metridium senile</i>	Basilar	0.7 (0.7, 0.7) (2)	5.0 (4.5, 5.5) (2)
	Sphincter	1.1 (1.1, 1.1) (2)	5.2 (4.7, 5.7) (2)
Annelida (Polychaeta) <i>Aphrodite aculeata</i>	Longitudinal	<0.1 (3)	8.7 (5.7, 11.7) (2)
	Dorsal longitudinal	<0.1 (3)	18.0 (17.4–18.9) (3)
Mollusca (Bivalvia) <i>Pecten maximus</i>	Phasic adductor	<0.1 (5)	13.4 (9.0–15.4) (3)
	Catch adductor	<0.1 (5)	11.9 (10.5–13.3) (2)
<i>Chlamys varius</i>	Phasic adductor	<0.1 (2)	16.8 (14.0–19.8) (3)
	Catch adductor	<0.1 (2)	—
<i>Modiolus modiolus</i>	Phasic adductor	1.1 (0.9–1.6) (4)	70.1 (64.5, 75.7) (2)
	Catch adductor	0.7 (0.4–1.2) (4)	39.6 (31.4, 47.7) (2)
<i>Glycimeris glytmeris</i>	Phasic adductor	0.2 (0.1–0.2) (3)	15.9 (13.4, 18.3) (2)

Table 5—continued

Animal	Muscle	Enzyme activities ($\mu\text{mol}/\text{min}$ per g fresh wt. of muscle at 25°C)	
		Phosphoenolpyruvate carboxykinase	Nucleoside diphosphate kinase
<i>Venus striatula</i>	Phasic adductor	<0.1 (4)	—
	Catch adductor	<0.1 (4)	—
<i>Ostrea edulis</i>	Phasic adductor	1.5 (1.0–2.2) (3)	119 (112–126) (3)
	Catch adductor	0.2 (0.1–0.2) (5)	56.5 (56, 57) (2)
<i>Mya arenaria</i>	Adductor	1.3 (0.7–2.0) (6)	159 (158, 160) (2)
<i>Ensis ensis</i>	Pedal retractor	<0.1 (2)	51.4 (50.0, 52.8) (2)
(Gastropoda)			
<i>Monodonta turbinata</i>	Pedal retractor	1.3 (1.0–1.7) (5)	70.7 (63.8, 78.1) (2)
<i>Littorina littorea</i>	Pedal retractor	1.9 (1.5–2.8) (3)	91.3 (82.4, 100) (2)
<i>Monodonta lineata</i>	Pedal retractor	1.5 (1.2–1.6) (3)	40.3 (39.8, 40.8) (2)
<i>Buccinum undatum</i>	Radular retractor	<0.1 (3)	39.7 (35, 44.3) (2)
<i>Patella vulgata</i>	Radular retractor	<0.1 (2)	47.6 (46.6, 48.6) (2)
<i>Murex trunculus</i>	Radular retractor	<0.1 (2)	—
Arthropoda (Crustaceae)			
<i>Homarus vulgaris</i>	Deep abdominal flexor	1.3 (0.5–2.3) (4)	28.6 (27.5–29.6) (2)
	Claw adductor	0.6 (0.6, 0.6) (2)	17.2 (16.1, 18.4) (2)
<i>Galathea squamifera</i>	Abdominal flexor	0.3 (0.2–0.4) (3)	27.4 (25.2, 29.6) (2)
<i>Crangon allmanni</i>	Abdominal flexor	<0.1 (3)	33.7 (32.4, 35.0) (2)
<i>Portunus puber</i>	Leg	<0.1 (3)	53.2 (50.6, 55.7) (2)
<i>Cancer pagurus</i>	Claw adductor	—	28.8 (26.4, 31.2) (2)
<i>Lepas anatifera</i>	Closer muscle	0.8 (0.5–1.2) (5)	59.4 (60.1, 58.7) (2)
(Chelicerata)			
<i>Limulus polyphemus</i>	Leg	1.4 (1.1–1.6) (3)	25.0 (23.4, 26.6) (2)
Echinodermata (Echinoidea)			
<i>Echinus esculentus</i>	Lantern retractor	<0.1 (3)	9.4 (9.0, 9.8) (2)
(Holothuroidea)			
<i>Thyone</i> sp.	Pharyngeal retractors	0.2 (0.1–0.3) (3)	1.0 (1.0, 1.1) (2)

adductor muscles of some bivalves (e.g. sand gaper, oyster, horse mussel), whereas adductor muscles of other bivalves contain lower activities of this enzyme (<0.1–0.2 $\mu\text{mol}/\text{min}$ per g of muscle, in scallops, striped venus and *Glycimeris*). In general, molluscan

muscles that contain the higher activities of phosphoenolpyruvate carboxykinase also contain high activities of nucleoside diphosphate kinase (40.3–119 $\mu\text{mol}/\text{min}$ per g for pedal retractor of the thick top shell and the phasic adductor of the oyster respect-

ively). The greater activity of the diphosphate kinase compared with the carboxykinase (approx. 100-fold) probably reflects the difference between near- and non-equilibrium reactions (see Crabtree & Newsholme, 1975; Newsholme & Crabtree, 1976). (The non-equilibrium nature of the carboxykinase reaction is indicated by the fact that, in general, the maximum activity of this enzyme is similar to the maximum glycolytic flux as indicated by the activities of phosphofructokinase; cf. Tables 3 and 5.) The activities of the carboxykinase and nucleoside diphosphate kinase are approx. 1.5 and 70 $\mu\text{mol}/\text{min per g}$ in the pedal retractor muscles of the gastropods (Table 5), which suggests that the succinate pathway is important for energy formation in these muscles. However, the radular retractor muscles of the gastropods contain very low activities of the carboxykinase ($<0.1 \mu\text{mol}/\text{min per g}$) but high activities of the nucleoside diphosphate kinase (approx. 40 $\mu\text{mol}/\text{min per g}$). Since these muscles appear to rely on aerobic metabolism for ATP generation, the high nucleoside diphosphate kinase activities may be related to the phosphorylation of ADP by the GTP formed in the tricarboxylic acid cycle.

In the abdominal muscle of the lobster and the leg muscle of the horse-shoe crab, the activities of the

carboxykinase are 1.3 and 1.4 $\mu\text{mol}/\text{min per g}$ respectively, but the diphosphate kinase activities are not as high as in other muscles (28.6 and 25.0 $\mu\text{mol}/\text{min per g}$ respectively; see Table 5). This suggests that the carboxykinase is not involved in the succinate pathway in these muscles. It is possible that it plays a similar role to the enzyme in vertebrate skeletal muscles (see Crabtree *et al.*, 1972). Further, in the adductor muscles of the bivalves, the activities of the carboxykinase are similar to the maximum glycolytic flux (cf. results in Tables 3 and 5), so that most of the phosphoenolpyruvate produced from glycolysis may enter the succinate pathway. In the abdominal muscles of lobster and leg muscles of horse-shoe crab, the activities of the carboxykinase (about 1.3 $\mu\text{mol}/\text{min per g}$) are considerably lower than the maximum glycolytic flux (about 10 $\mu\text{mol}/\text{min per g}$), which is similar to the situation in vertebrate muscles.

Importance of arginine phosphate as a source of energy in muscles of the marine invertebrates

The activities of arginine kinase in the muscles investigated range from 24 to 1754 $\mu\text{mol}/\text{min per g}$ (basilar muscle of the sea-anemone and abdominal flexor of the lobster respectively; see Table 6). There is evidence that arginine kinase catalyses a near-

Table 6. *Maximum activities of arginine kinase in muscles of some marine invertebrates*

The activities were measured as described in the Materials and Methods Section and they are presented as means; the ranges and the numbers of separate animals used are given in parentheses. Common names are given in Table 3.

Animal	Muscle	Arginine kinase activity ($\mu\text{mol}/\text{min per g}$ fresh wt. of muscle at 25°C)
Coelenterata (Anthozoa)		
<i>Metridium senile</i>	Basilar	24 (20.0–26.0) (3)
Mollusca (Bivalvia)		
<i>Pecten maximus</i>	Phasic adductor	930 (872–977) (4)
	Catch adductor	142 (102–197) (4)
<i>Chlamys varius</i>	Phasic adductor	900 (836–966) (3)
	Catch adductor	140 (130–156) (4)
<i>Modiolus modiolus</i>	Phasic adductor	303 (250–342) (3)
	Catch adductor	54 (50–62) (4)
<i>Ostrea edulis</i>	Phasic adductor	147 (117–220) (3)
	Catch adductor	55 (45–89) (3)
<i>Ensis ensis</i>	Pedal retractor	1235 (850–1510) (3)
(Gastropoda)		
<i>Monodonta turbinata</i>	Pedal retractor	620 (503–740) (3)
<i>Littorina littorea</i>	Pedal retractor	1020 (1000, 1040) (2)
<i>Monodonta lineata</i>	Pedal retractor	782 (725–802) (3)
<i>Patella vulgata</i>	Radular retractor	276 (251–342) (4)
<i>Loligo forbesi</i>	Mantle	961 (830–1120) (3)
	Fin	830 (800–881) (3)
Arthropoda (Crustacea)		
<i>Homarus vulgaris</i>	Abdominal flexor	1754 (1482–2054) (4)
	Claw adductor	714 (688, 740) (2)
<i>Galathea squamifera</i>	Abdominal flexor	1620 (1405, 1835) (2)
<i>Pachygrapsus marmoratus</i>	Leg	1250 (960–1400) (3)
<i>Portunus puber</i>	Leg	1470 (1220–1685) (3)

equilibrium reaction in these muscles (Beis & News-holme, 1975), so that the maximum activity of this enzyme will greatly exceed the maximum flux through the reaction. Consequently, the activities provide only a qualitative indication of the maximum rate of ATP formation from arginine phosphate. Nonetheless, the activities of arginine kinase in some muscles are very high in comparison with other enzymes that catalyse other near-equilibrium reactions. The activities of lactate dehydrogenase in lobster abdominal muscle and claw muscle of the edible crab are 127 and 120, whereas arginine kinase activity is 1754 and 1905 $\mu\text{mol/min per g}$ respectively (cf. data in Tables 4 and 6). The highest activities of arginine kinase are found in some muscles that have a maximum glycolytic flux considerably lower than would be required to generate sufficient ATP to support maximum contractile activity: in the phasic adductor muscle of the scallop, the pedal retractor muscle of the razor clam, the mantle muscle of the squid and the abdominal flexor muscles of the two lobsters, the maximum ATP requirement is about twice that which could be generated from anaerobic glycolysis (see Table 2) and the arginine kinase activities are 930, 1235, 961, 1620 and 1754 $\mu\text{mol/min per g}$ respectively. It is suggested that in these muscles arginine phosphate breakdown is as important as carbohydrate utilization in the energy formation for the contractile process. The high concentrations of arginine phosphate reported in the abdominal muscle of the lobster and the phasic adductor muscle of the scallop (Beis & News-holme, 1975) are consistent with this suggestion. Nonetheless, high activities of arginine kinase are also found in the other muscles (e.g. claw adductor of the lobster, fin muscle of the squid), in which it is suggested that arginine phosphate breakdown supplements the energy produced via glycolysis. The low activities of the phosphokinase in the catch muscles of bivalve molluscs are expected since, even during contractile activity, these muscles have a low rate of energy utilization (Rüegg, 1971). However, in the phasic adductor muscle of the oyster, the maximum rate of glycolysis could only provide about 25% of the energy required for contraction (Table 2), but the activity of arginine kinase is relatively low in these muscles (Table 6). Since the aerobic capacity of this muscle is very low (Alp *et al.*, 1976), it is suggested that an alternative fuel (as yet unknown) is present in these muscles.

The rephosphorylation of ADP by arginine phosphate produces arginine. In some muscles that possess high activities of arginine kinase, the activities of octopine dehydrogenase, which utilizes arginine as a substrate, are also high (e.g. phasic adductor of scallops, pedal retractor muscles of razor clam, fin and mantle muscles of squids; see Table 4). The advantage of octopine formation in these muscles may be that NADH re-oxidation will result in the

removal of arginine (to form octopine), which will facilitate the utilization of arginine phosphate to form ATP. It is considered that creatine phosphate breakdown is favoured in vertebrate muscle during contraction by an increase in the concentration of H^+ (Sahlin *et al.*, 1975). In these invertebrate muscles, in which arginine phosphate may play a significant quantitative role in energy generation, an additional effect may be obtained from the presence of octopine dehydrogenase.

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